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(54) INHIBITOR PREPARATION OF ANGIOTENSIN CONVERTING ENZYME

(57)Abstract:

PROBLEM TO BE SOLVED: To obtain the subject preparation excellent in inhibiting activity on angiotensin converting enzyme, high in safety and useful as a pharmaceutical product for the prophylaxis, treatment, etc., of hypertension, a food, etc., by compounding Leu-Pro-Pro and an active component.

SOLUTION: γ-Zein is dispersed in distilled water, the dispersion is adjusted to pH 12 with 1N NaOH to dissolve the γ-zein, then the resultant solution is heated at 100°C for 30min to denature the γ-zein, and subsequently thermolysin is added to the mixture, and the reaction is stopped with 1N HCl. The reaction mixture is subjected to HPLC(high performance liquid chromatography) to collect Val-His-Leu-Pro-Pro-Pro, the obtained peptide is treated with leucine aminopeptidase to lead to Leu-Pro-Pro-Pro, and then the product is hydrolyzed at 100°C for 10min after adding 12N HCl to obtain Leu-Pro-Pro. Subsequently, the resultant Leu-Pro-Pro is compounded as an active component to obtain a preparation, and thus the objective enzyme inhibitor preparation having excellent inhibiting activity on angiotensin converting enzyme, extremely high in safety and useful as a pharmaceutical product, a functional food, etc., is obtained.

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* NOTICES *

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] Especially this invention relates to the angiotensin conversion enzyme inhibitor with which it is expected that it can use for drugs or food useful for the prevention and the therapy of hypertension which it is in the inclination of an increment in recent years, and are expected the cure about angiotensin conversion enzyme inhibitor.
[0002]
[Description of the Prior Art] Although it is well known by the onset of hypertension that the renin-angiotensin series has deep relation, the role with angiotensin converting enzyme (it is also called EC3.4.15.1 and Following ACE) important for this renin-angiotensin series is played. In this case, the angiotensinogen secreted by the liver acts to angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) which was decomposed by the enzyme renin produced by the kidney, and ACE transforms this thing to angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). And this angiotensin II shrinks a blood vessel wall smooth muscle, raises blood pressure, acts on the adrenal cortex further and has an operation of promoting secretion of aldosterone. Moreover, although the bradykinin whose pressure the enzyme kallikrein which exists in plasma disassembles the protein called kininogen, make extend a blood vessel, and is made to lower is produced, it will be decomposed by operation of ACE and this bradykinin will be inactivated. Thus, ACE disassembles a pressure-lowering nature peptide (bradykinin) on the other hand, and advances blood pressure towards a rise as a result while it produces a pressure-up nature peptide (angiotensin II) in one side. Therefore, it is possible by controlling this enzyme activity to prevent elevation of blood pressure (pressure lowering).
[0003] Much synthetic material, such as captopril (D-2-methyl-3-mercapto propanoyl-L-proline), is known by making into the start several sorts of peptide nature inhibitors obtained from snake venom as ACE inhibitor, among these practical use is already presented with captopril as an oral hypotensor. Moreover, ACE inhibitor is found out also in a microorganism or various food, and the utilization as a hypotensor is considered in recent years.
[0004] Moreover, the ACE inhibitor of the trypsin hydrolyzate origin of cow's milk casein is isolated, or it processes by the peptidase further, and using this as an antihypertensive is proposed (JP,60-23085,B, 60-23086, 60-23087, JP,61-36226,A, 61-36227). Moreover, recently, using the hydrolyzate by the serine protease of the Bacillus bacteria origin of fishes protein or soybean protein, the metal protease of the Bacillus bacteria origin, or the thiol protease of the vegetable origin as an antihypertensive is proposed (JP,62-169732,A).
[0005] The prolamin one side and whose corn protein are principal components about a prolamin, including 50 - 60% and a glutelin 35 to 40% is called a zein (zein). A zein is divided into three sorts, alpha, beta, and gamma, (J. Cereal Sci.5,117 (1987)). In gamma-zein, the repeat structure which makes a base unit Val-His-Leu-Pro-Pro-Pro is included (Nucleic Acids Res.13(5), 1493 (1985)).
[0006]
[Problem(s) to be Solved by the Invention] Angiotensin conversion enzyme inhibitor is always called for for new useful antihypertensive ******. Moreover, it is these days when the functional food which aims at prevention of various symptoms, such as hypertension, etc. through everyday intake only as drugs is also called for. Therefore, it aims at offering specific oligopeptide system angiotensin conversion enzyme inhibitor this invention having the outstanding angiotensin conversion enzyme inhibition operation and the outstanding blood-pressure descent operation, high [this invention / very] safety and usable also as functional food only as drugs.
[0007]
[Means for Solving the Problem] this invention persons found out that the fixed peptide obtained by hydrolyzing gamma-zein in the corn protein which is the cheap and most common food-grade protein by the specific protease has angiotensin conversion enzyme inhibition activity, and that the above-mentioned technical problem was solvable by

using this peptide, as a result of searching various matter which has ACE inhibition activity. That is, this invention offers the angiotensin conversion enzyme inhibitor which contains Leu-Pro-Pro as an active principle.

[0008]

[Embodiment of the Invention] according to a conventional method, it is separable from the zein protein separated from the corn protein which obtains that it is a gamma-zein used for this invention, and is obtained in a sorghum or the manufacture process of corn starch (for example, Plant Physiol., 80,623 (1986)). Moreover, the example of preparation of gamma-zein is shown in the example 1 of reference.

[0009] Hydrolysis in this invention is performed by the following process.

a) Give gamma-zein to thermolysin hydrolysis first and it makes Val-His-Leu-Pro-Pro-Pro generate. That is, gamma-zein is first dissolved in alkali solutions, such as a sodium-hydroxide water solution, and the low-molecular impurity solubilized with the ultrafiltration is removed. Subsequently, thermolysin is made easy to carry out processing for 5 minutes to 1 hour if needed at pH 10-12 and the temperature of 80-100 degrees C etc., to denature gamma-zein, and to commit. Under the present circumstances, the fraction which carried out depolymerize is removed with an ultrafiltration.

[0010] Subsequently, a hydrochloric acid etc. adjusts pH near neutrality, calcium content buffer solution adjusts pH to 6-9, temperature is kept at 30-80 degrees C, thermolysin is added, and an enzyme reaction is made to perform for 1 to 40 hours. As the buffer solution, it is 0.005-0.01M. The CaCl content 0.05M tris hydrochloric-acid buffer solution (pH 8-8.5) etc. is used suitably. 0.1 - 10 weight section is suitable for the amount of the thermolysin used to the substrate 100 weight section. A reaction is terminated by adding acids, such as a hydrochloric acid, and carrying out deactivation of the enzyme as three or less pH.

[0011] The low-molecular content filtrate which gives the enzyme liquid after a reaction to an ultrafiltration, and is passed is collected. A Val-His-Leu-Pro-Pro-Pro content solution is obtained by condensing, giving a column chromatography, for example, sephadex LH-20 column chromatography, after neutralizing filtrate, in alkali water solutions, such as a sodium hydroxide, and comparing the elution pattern by HPLC of each fraction with it of synthetic Val-His-Leu-Pro-Pro-Pro. A column chromatography, for example, an SP-Toyo Perl 650S cation-exchange chromatography, the opposition system HPLC, etc. can refine this thing further.

[0012] b) Next, hydrolyze Val-His-Leu-Pro-Pro-Pro by the leucine aminopeptidase, and make His-Leu-Pro-Pro-Pro or Leu-Pro-Pro-Pro generate. This enzyme reaction is usually performed at 30-60 degrees C among the buffer solution of pH 6-9 for 1 to 24 hours. As the buffer solution, a 0.05MMgCl content 0.1M tris hydrochloric acid (pH8.6) etc. is used. 0.1 - 10 weight section is suitable for the amount of the leucine aminopeptidase used to the substrate 100 weight section.

[0013] At 100 degrees C, a reaction is heated for 5 minutes and terminated. A column chromatography HPLC, for example, an opposition system etc., can perform isolation purification of the specified substance from reaction termination liquid. The comparison of the HPLC elution pattern of a synthetic peptide can perform a trace of the quality of the specified substance like the case of a.

[0014] c) Remove one edge Pro C end each by hydrolyzing Val-His-Leu-Pro-Pro-Pro, His-Leu-Pro-Pro-Pro, or Leu-Pro-Pro-Pro by Carboxypeptidase C next, or giving mild acidolysis. This enzyme reaction is usually performed at 30-60 degrees C among the buffer solution of pH 4-7 for 1 to 24 hours. 0.1M citrate buffer solution etc. is used as the buffer solution. 0.1 - 10 weight section is suitable for the amount of the carboxypeptidase C used to the substrate 100 weight section. At 100 degrees C, a reaction carries out heating for 5 minutes etc., and is terminated.

[0015] Acidolysis is usually performed at the temperature of 80-120 degrees C for 5 to 120 minutes using acids, such as a hydrochloric acid of 0.1 to concentration 6 convention. A reaction is terminated by neutralizing in a sodium-hydroxide water solution etc. In any case, a column chromatography HPLC, for example, an opposition system etc., can perform isolation purification of the specified substance from reaction termination liquid. The comparison of the HPLC elution pattern of a synthetic peptide can perform a trace of the quality of the specified substance like the case of a. In addition, the process of Above b is performed if needed. Moreover, when performing both the processes of b and c, any may be performed first.

[0016] The peptide of the amino acid degrees of polymerization 3-5 acquired by the above shows ACE inhibition activity. When using these peptides as an ACE inhibitor, these peptides may be contained independently, and may be contained as mixture of the rate of arbitration, and may contain other peptides of the hydrolyzate origin, and amino acid as a minor component further.

[0017] the peptide concerned -- as it is -- or it is usually used by making it at least one pharmaceutic aid and pharmaceutical preparation constituent. The mammals including Homo sapiens can be medicated with the peptide of this invention parenterally (namely, an intravenous injection, rectum administration, etc.) or in taking orally, and it can

be manufactured in the gestalt suitable for each medication method.
[0018] The formulation as injections usually includes a sterilized water water solution. The pharmaceutical preparation of the above-mentioned gestalt can contain other medicine manufacture adjuvants other than water (methyl parahydroxybenzoate, propyl para hydroxybenzoate, etc.), such as a buffer and pH regulators (dibasic sodium phosphate, citric acid, etc.), isotonizing agents (a sodium chloride, glucose, etc.), and a preservative, again. This pharmaceutical preparation can be sterilized with filtration, mixing of the germicide to a constituent, and the exposure and heating of a constituent that let a bacteria maintenance filter pass. this medicine manufacture -- again -- as a sterilization solid-state constituent -- manufacturing -- business -- the time -- sterilized water etc. -- dissolving -- it can also be used .
[0019] An internal use agent manufactures medicine in the form of having been suitable for absorption by the gastroenteric organ. A tablet, a capsule, a granule, a fine grain agent, and powders can include pharmaceutic aid (syrup, gum arabic, gelatin, sorbitol, tragacanth, a polyvinyl pyrrolidone, hydroxypropylcellulose, etc.) in ordinary use, for example, binders, excipients (a lactose, a sugar, corn starch, calcium phosphate, sorbitol, glycine, etc.), lubricant (magnesium stearate, talc, a polyethylene glycol, silica, etc.), disintegrator, and wetting agents (potato starch, carboxymethyl cellulose, etc.) (sodium lauryl sulfate etc.). A tablet can be coated with a conventional method. Oral liquids and solutions can be used as a water solution etc., or can be used as a dry product. Such oral liquids and solutions may include, additives, for example, preservatives, in ordinary use (methyl p-hydroxybenzoate or propyl, sorbic acid, etc.).
[0020] Although various amounts of this peptide in the ACE inhibitor of this invention are changeable, 1 - 100% (w/w) is usually suitable. 0.5-500mg/kg/day is suitable for the dose of this ACE inhibitor as an active principle. In addition, each acute toxicity of the peptide of this invention is LD(rat, internal use) >5 g/kg.
[0021] Moreover, the peptide of this invention may be eaten as the functional food which added nutrients remaining as it is or various etc., or was made to contain in an eating-and-drinking article, and gave the function of a blood-pressure descent operation and hypertension prevention from having the advantage which does not have a bad influence on a living body even if it takes in so much, and health food. namely, nutrients, such as for example, various vitamins and minerals, -- in addition, food and the solid food article of various configurations with liquefied nutrition supplement drink, soybean milk, soup, etc. -- it is as it is as still more powdered -- it is -- it can also add and use to various food. The content of this peptide in the ACE inhibitor of this invention as this functional food and health food and intake are the same also in the above-mentioned medicine manufacture, and good.
[0022]
[Example] Next, an example explains this invention. % shows weight % among an example.
Example 1 Preparation of each oligopeptide, and ACE inhibition activity a 25ml of distilled water is made to distribute preparation gamma-zein 0.5g of Val-His-Leu-Pro-Pro-Pro, and it is 1N. It adjusted to pH12 by NaOH, and gamma-zein was dissolved. Subsequently, the ultrafiltration using Amicon PM-10 (cut off molecular weight 10,000) as ultrafiltration membrane was given, and the solubilized low-molecular impurity was removed. NaOH of pH12 was added to inner liquid, and it considered as 25ml of whole picture, and heated at 100 degrees C for 30 minutes, and gamma-zein was denatured. In order to remove the fraction which carried out depolymerize by this processing, the again same ultrafiltration as the above is given, and distilled water is added to inner liquid, and it considers as 25ml of whole picture, and is 1 moreN. It was made neutrality by HCl.
[0023] It is 0.05M of 0.25 ** to the whole picture. After adding the CaCl content 0.25M tris HCl buffer solution (pH8.5) and keeping at 37 degrees C, thermolysin (sigma company) 18mg was added. 40 hours after, 1N It adjusted to pH1.7 by HCl, the reaction was stopped, and it collected low-molecular [which give to the same ultrafiltration as the above and it passes]. It is 1N about this. The condensed concentration liquid was added in the column of sephadex LH-20 after neutralization by NaOH, and elution was carried out with distilled water (elution conditions: column height of 70cm, the bore of 1.6cm, the sample addition of 2ml, rate-of-flow 33 ml/hr).
[0024] the elution pattern by HPLC was investigated using the small quantity of each fraction, and fractions with the peak of the elution location which synthetic Val-His-Leu-Pro-Pro-Pro shows, and homotopic were collected (elution condition [of HPLC]: -- column Waters Radial PAK [ ] -- C-8, 10 micrometers, sample addition 5microl, rate-of-flow 1 ml/min, elution phosphate buffer solution (10mM KHPO, 50mM NaSO, pH3.0):acetonitrile =2:3, and detection UV210nm).
[0025] Fractions with the peak by which adds in SP-Toyo Perl 650S column which equilibrated this fraction with 5mM buffer solution (pH4.0), and is eluted in the linear density gradient of 0 - 0.3M NaCl, and elution is carried out to synthetic Val-His-Leu-Pro-Pro-Pro and homotopic in HPLC were collected (SP-Toyo Perl elution conditions: column height of 20cm, the bore of 1.6cm, rate-of-flow 100 ml/hr, 0 containing the elution 5mM acetic-acid buffer solution

(pH4.0) - 0.3M NaCl.). The elution conditions of HPLC are the same as the above.
[0026] Opposition silica gel column SepPAK which gave HPLC, isolated preparatively only the peak of synthetic Val-His-Leu-Pro-Pro-Pro and homotopic after condensing the collected fraction, and was washed by HCl of pH2 It was made to stick to C-18 (Waters), and after removing the salt intermingled by HCl of pH2, elution was carried out with the methanol and amino acid analysis was performed (the HPLC elution conditions at the time of preparative isolation: only the sample addition of others is the same as that of the conditions in the beginning at 25microl). Amino acid analysis is 6Ns about a sample by the above. It dissolved in HCl and the amino acid analyzer after 24-hour heating performed at 110 degrees C under the vacuum.
[0027] The mole ratio which set Leu to 1 as a result is Val. 1.3 His 1.2 Leu1, Pro It was set to 3.1 and Val-His-Leu-Pro-Pro-Pro has been collected. Moreover, the result of mass analysis is 659 (M+1), and was in agreement with the anticipation molecular weight of the above-mentioned peptide.
[0028] b) It is 0.05M about the preparation leucine aminopeptidase (Boehringer Mannheim Yamanouchi) (liquefied [ml] 5mg /) of Leu-Pro-Pro-Pro. It dissolved in 800micro (pH8.6) of MgCl content 0.1M tris hydrochloric acids l, and considered as enzyme liquid. 300microM Val-His-Leu-Pro-Pro-Pro 50microl and 200micro of enzyme liquid l were mixed, and it was made to react at 37 degrees C for 23 hours. After the reaction, from reaction mixture, the peaks by which elution is carried out to synthetic Leu-Pro-Pro-Pro and homotopic by HPLC were collected, and amino acid analysis was performed (HPLC elution conditions: it is the same as the conditions in the beginning except having set to have set pH of the phosphate buffer solution to be used to 2.5, and a sample addition to 10microl). The mole ratio which set Leu to 1 as a result is Val. 0.16, Leu 1, His 0.19, Pro It was set to 2.51 and Leu-Pro-Pro-Pro has been collected.
[0029] c) Preparation 6.3mM of Leu-Pro-Pro Leu-Pro-Pro-Pro 200microl and 12Ns HCl200microl was mixed and the hydrolysis reaction was made to obey at 100 degrees C for 10 minutes. Subsequently, the fraction which has the peak (3.18mm) of synthetic Leu-Pro-Pro and homotopic by the elution by HPLC was recoverable. ((Elution conditions: Co.) Column Merck Lichrosorb RP-SelectB 5micrometer, rate-of-flow 1 ml/min, elution Phosphate buffer solution (pH2.5):acetonitrile =5:1, detection UV210nm) .
[0030] d) It obtained like Above c from preparation Val-His-Leu-Pro-Pro-Pro of Val-His-Leu-Pro-Pro.
[0031] e) The ACE inhibition activity of each peptide which is beyond measurement of ACE inhibition activity, and was made and obtained was measured as the following. That is, first, 5g rabbit langue acetone powder was melted to the 50ml 0.1M sodium-borate buffer solution (pH8.3), centrifugal processing was carried out under the conditions for 40,000 G or 40 minutes, the supernatant liquor was further diluted with the above-mentioned buffer solution 5 times, and angiotensin-converting-enzyme liquid was obtained.
[0032] 0.03ml of each peptide solution is put into a test tube, and the 0.25ml HIPURIRU histidyl leucine (the last concentration -- it contains NaCl 300mM 5 mM) was added to this, subsequently the 0.1ml of the above-mentioned angiotensin-converting-enzyme liquid was added to it, and it was made to react to it for 30 minutes at 37 degrees C as a substrate Then, after adding 0.25ml of 1-N hydrochloric acids and stopping a reaction, 1.5ml ethyl acetate was added, the 228nm absorption value of the HIPURIRU acid extracted in ethyl acetate was measured, and this was made into enzyme activity. In addition, the absorption value of 228nm in case this invention inhibitor is not included on this condition was about 0.35. Such an experiment of two or more lines was, and the rate of inhibition was computed from the following formula.
[0033]
[Formula 1]

$$\text{阻害率} = \frac{A-B}{A} \times 100 \quad (\%)$$

[0034] A: 228nm absorption value B in case an inhibitor is not included : it asked for the inhibitor concentration I at the time of 228nm absorption value in inhibitor addition, and 50% of rates of inhibition. A result is shown in Table 1.
[0035]
[Table 1]

	$I_{50}(\mu M)$	備考
Leu – Pro – Pro	9.8	本発明
Val – His – Leu – Pro – Pro	18	比較例
Val – His – Leu – Pro – Pro – Pro	200	〃
Leu – Pro – Pro – Pro	(1mMで33%阻害)	〃

[0036] Example 2 The Wistar system male rat (Japanese Rat, one groups [five]) with a blood-pressure descent operation weight [of Leu-Pro-Pro] of 200g was anesthetized by urethane 1.5 g/kg intraperitoneal administration, and the total neck arterial blood pressure was continuously recorded through the transducer (SCK-590 and Nihon Kohden Corp.) according to the conventional method. From the leg vein, Leu-Pro-Pro dissolved in the physiological saline was prescribed for the patient, angiotensin I (Homo sapiens array, sigma company) 100 ng/kg was repeated and prescribed for the patient after 5, 15, 25, and 35 minutes of those, and change of the mean blood pressure of order was measured. As contrast, what prescribed the physiological saline for the patient was used. A result is shown in Table 2. This peptide controlled effectively the pressure up by angiotensin I 5 minutes after administration, and, in addition, the operation was maintained also after 35 minutes.

[0037]

[Table 2]

試験群	血圧上昇（△mmHg）			
	5分後	15分後	25分後	35分後
対照（生理食塩水投与群）	21±3 22±1	27±6 22±8	29±11 23±6	32±8 22±5
Leu－Pro－Pro 40mg／kg投与群	16±6 15±5	24±5 23±5	25±4 21±7	23±3 22±4
Leu－Pro－Pro 125mg／kg投与群	8±1 9±3	15±6 14±5	16±5 16±4	17±4 16±3

上段：最高血圧　下段：平均血圧

[0038] Example 3 Intravenous injection agent Leu-Pro-Pro is dissolved in a 20 to 100 times (volume/weight) as many sterilization physiological saline as this, and let the filtrate filtered with the filter (0.45 micrometers of apertures) in sterile be injections.

[0039] Example 4 Tablet Leu-Pro-Pro 7 section hydroxypropylcellulose 1 section lactose 10.9 section potato starch 1 section magnesium stearate The ethanol water-solution 20 section is prepared 60% containing the 0.1 section hydroxypropylcellulose 1 section. After adding the this peptide 7 section and the lactose 10.9 section and fully kneading, under reduced pressure, it dries, and the potato starch 1 section and the magnesium stearate 0.1 section are added, and it mixes with them at the obtained dry matter, and tablets with a tableting machine.

[0040] Example 1 of reference It carried out according to the approach (J. Cereal Sci.5,117 (1987)) of the preparation Esen of gamma-zein. It is 1% in 100g (cosmopolitan dent corn) of grinding corn. The amount containing 2-mercaptoethanol of 5 times as many 60% isopropyl alcohol water as this was applied, and all zein fractions were extracted by stirring at 60 degrees C for 2 hours. Centrifugal separation of the mixture was carried out by 3,000G for 10 minutes, distilled water of isochore and 3M sodium acetate water solution of 0.02 ** were added to supernatant liquid, and it doubled with pH6 with a small amount of acetic acid, it put at 4 degrees C overnight, and alpha and beta-zein was settled. Subsequently, centrifugal separation was carried out by 3,000G for 10 minutes, and supernatant liquid was freeze-dried, and little distilled water was distributed, the dry matter was dialyzed to distilled water using the dialysis tube, subsequently it freeze-dried, and gamma-zein 0.4g was obtained as light yellow powder.

[0041]

[Effect of the Invention] According to this invention, the ACE inhibitor which has the outstanding ACE inhibitory action and the outstanding blood-pressure descent operation is offered. Even if it takes in the ACE inhibitor of this invention in large quantities for the food protein origin, its safety is very high, therefore it does not show a side effect.

[Translation done.]

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CLAIMS

[Claim(s)]
[Claim 1] Angiotensin conversion enzyme inhibitor which contains Leu-Pro-Pro as an active principle.

[Translation done.]

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(54)【発明の名称】　アンジオテンシン変換酵素阻害剤

(57)【要約】

【課題】　優れたアンジオテンシン変換酵素阻害作用を有し、安全性が極めて高く、医薬品としてのみならず機能性食品としても使用可能な特定のオリゴペプチド系アンジオテンシン変換酵素阻害剤の提供。

【解決手段】　Ｌｅｕ－Ｐｒｏ－Ｐｒｏを有効成分として含有するアンジオテンシン変換酵素阻害剤。

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【特許請求の範囲】

【請求項1】 Leu-Pro-Proを有効成分として含有するアンジオテンシン変換酵素阻害剤.

【発明の詳細な説明】

【0001】

【発明の属する技術分野】本発明はアンジオテンシン変換酵素阻害剤に関し、特に近年増加の傾向にあり対策が望まれている高血圧症の予防及び治療に有用な医薬品又は食品に利用できることが期待されるアンジオテンシン変換酵素阻害剤に関するものである.

【0002】

【従来の技術】高血圧症の発症にはレニン-アンジオテンシン系が深いかかわりを有していることがよく知られているが、このレニン-アンジオテンシン系にはアンジオテンシン変換酵素（EC3.4.15.1、以下ACEとも言う）が重要な役割を果たしている.この場合ACEは、肝で分泌されるアンジオテンシノーゲンが腎で産生される酵素レニンにより分解されたアンジオテンシンI（Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu）に対して作用し、このものをアンジオテンシンII（Asp-Arg-Val-Tyr-Ile-His-Pro-Phe）に変換させる.そして、このアンジオテンシンIIは血管壁平滑筋を収縮させて血圧を高め、さらに副腎皮質に作用してアルドステロンの分泌を促進させるなどの作用を有する.また、血漿に存在する酵素カリクレインはキニノーゲンと呼ばれる蛋白質を分解し、血管を拡張させ降圧させるブラジキニンを産生するが、このブラジキニンはACEの作用により分解され、不活性化されてしまう.このように、ACEは一方で昇圧性ペプチド（アンジオテンシンII）を生じさせるとともに、他方で降圧性ペプチド（ブラジキニン）を分解し、結果として、血圧を上昇の方向に進める.したがってこの酵素活性を抑制することによって血圧上昇を防ぐこと（降圧）が可能である.

【0003】ACE阻害物質としては蛇毒より得られた数種のペプチド性阻害剤を初めとして、カプトプリル（D-2-メチル-3-メルカプトプロパノイル-L-プロリン）などの合成物質が多数知られており、このうちカプトプリルは経口降圧剤として既に実用に供されている.また、近年、微生物あるいは種々の食品中にもACE阻害物質が見出され、降圧剤としての実用化が検討されている.

【0004】また、牛乳カゼインのトリプシン加水分解物由来のACE阻害物質を単離し、あるいはさらにペプチダーゼで処理し、これを血圧降下剤として用いることが提案されている（特公昭60-23085号、同60-23086号、同60-23087号、特開昭61-36226号、同61-36227号）.また最近では、魚類タンパク質または大豆タンパク質のバチルス属細菌由来のセリンプロテアーゼ、バチルス属細菌由来の金属プロテアーゼまたは植物由来のチオールプロテアーゼによる加水分解物を血圧降下剤として用いることが提案されている（特開昭62-169732号）.

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【0005】一方、とうもろこしタンパク質はプロラミンを50～60%、グルテリンを35～40%含み、主成分であるプロラミンはゼイン（zein）と呼ばれる.ゼインはα、β、γの3種に分けられる（J.Cereal Sci.5,117(1987)）.γ-ゼイン中にはVal-His-Leu-Pro-Pro-Proを基本単位とする繰り返し構造が含まれている（Nucleic Acids Res.13(5),1493(1985)）.

【0006】

【発明が解決しようとする課題】新規有用な血圧降下剤ひいてはアンジオテンシン変換酵素阻害剤は常に求められている.また医薬品としてのみならず、日常の摂取を通して高血圧等の種々の症状の予防等を図る機能性食品も求められる昨今である.従って本発明は優れたアンジオテンシン変換酵素阻害作用ならびに血圧降下作用を有し、安全性が極めて高く、医薬品としてのみならず機能性食品としても使用可能な特定のオリゴペプチド系アンジオテンシン変換酵素阻害剤を提供することを目的とする.

【0007】

【課題を解決するための手段】本発明者らはACE阻害活性を有する物質を種々検索した結果、安価で最も一般的な食品用タンパク質であるとうもろこしタンパク質中のγ-ゼインを特定のプロテアーゼで加水分解して得られる一定のペプチドがアンジオテンシン変換酵素阻害活性を有すること、及び該ペプチドを用いることによって上記課題を解決できることを見出した.すなわち本発明はLeu-Pro-Proを有効成分として含有するアンジオテンシン変換酵素阻害剤を提供する.

【0008】

【発明の実施の形態】本発明に使用するγ-ゼインとしてはとうもろこし、またはコーンスターチの製造過程で得られるとうもろこしタンパク質から分離したゼインタンパク質から、常法に従って分離することができる（例えばPlant Physiol.,80,623(1986)）.また参考例1にγ-ゼインの調製例を示す.

【0009】本発明における加水分解は次の工程によって行われる.

a） γ-ゼインはまずサーモライシン加水分解に付してVal-His-Leu-Pro-Pro-Proを生成させる.すなわちまずγ-ゼインを水酸化ナトリウム水溶液等のアルカリ溶液に溶解し、限外沪過により可溶化した低分子夾雑物を除去する.ついで必要に応じpH10～12、温度80～100℃で5分～1時間処理

する等してゥーゼインを変性させ、サーモライシンを働きやすくする。この際低分子化した画分は限外沪過により除く。

【0010】ついでpHを塩酸等で中性付近に調整し、Ca含有緩衝液でpHを6～9に調整し、温度を30～80℃に保ち、サーモライシンを加え1～40時間酵素反応を行わせる。緩衝液としては0．005～0．01M CaCl含有0．05Mトリス塩酸緩衝液（pH8～8．5）等が好適に用いられる。サーモライシンの使用量は基質100重量部に対し0．1～10重量部が適当である。反応は例えば塩酸等の酸を添加してpH3以下として酵素を失活させることにより終了させる。

【0011】反応後酵素液を限外沪過に付して通過する低分子含有沪液を回収する。水酸化ナトリウム等のアルカリ水溶液で沪液を中和後、濃縮し、カラムクロマトグラフィー、例えばセファデックスLH-20カラムクロマトグラフィーに付し、各画分のHPLCによる溶出パターンを合成Val-His-Leu-Pro-Pro-Proのそれと比較することにより、Val-His-Leu-Pro-Pro-Pro含有溶液を得る。このものはカラムクロマトグラフィー、例えばSP-トヨパール650S陽イオン交換クロマトグラフィー、逆相系HPLCなどによりさらに精製することができる。

【0012】b）　次にVal-His-Leu-Pro-Pro-Proをロイシンアミノペプチダーゼで加水分解してHis-Leu-Pro-Pro-ProまたはLeu-Pro-Pro-Proを生成させる。この酵素反応は通常、pH6～9の緩衝液中、30～60℃で1～24時間行う。緩衝液としては0．05MMgCl含有0．1Mトリス塩酸（pH8．6）等を使用する。ロイシンアミノペプチダーゼの使用量は基質100重量部に対し、0．1～10重量部が適当である。

【0013】反応は例えば100℃で5分間加熱するなどして終了させる。反応終了液から目的物の単離精製はカラムクロマトグラフィー、例えば逆相系HPLCなどによって行うことができる。目的物質の追跡はa）の場合と同様合成ペプチドのHPLC溶出パターンの比較によって行うことができる。

【0014】c）　次にVal-His-Leu-Pro-Pro-Pro、His-Leu-Pro-Pro-ProまたはLeu-Pro-Pro-Proをカルボキシペプチダーゼで加水分解するか、温和な酸加水分解に付すことにより各C末端Proを1つ外す。この酵素反応は通常pH4～7の緩衝液中30～60℃で1～24時間行う。緩衝液としては0．1Mクエン酸緩衝液等を使用する。カルボキシペプチダーゼCの使用量は基質100重量部に対し0．1～10重量部が適当である。反応は例えば100℃で5分間加熱する等して終了させる。

【0015】酸加水分解は通常、濃度0．1～6規定の

塩酸等の酸を用い、温度80～120℃で5～120分行う。反応は水酸化ナトリウム水溶液等で中和することにより終了させる。いずれの場合も反応終了液から目的物の単離精製はカラムクロマトグラフィー、例えば逆相系HPLCなどによって行うことができる。目的物質の追跡はa）の場合と同様合成ペプチドのHPLC溶出パターンの比較によって行うことができる。なお、上記b）の工程は必要に応じ行う。またb）とc）の工程を共に行う場合いずれを先に行っても良い。

【0016】上記によって得られるアミノ酸重合度3～5のペプチドはACE阻害活性を示す。これらのペプチドをACE阻害剤として使用する場合、これらのペプチドは単独で含有されていても良く、また任意の割合の混合物として含有されていても良く、さらに加水分解物由来の他のペプチド、アミノ酸をマイナー成分として含有していても良い。

【0017】当該ペプチドはそのまま、または通常少なくとも1つの製剤補助剤と製剤組成物にして使用する。本発明のペプチドは非経口的（すなわち、静脈注射、直腸投与等）または経口的にヒトをはじめとする哺乳類に投与し、各投与方法に適した形態に製剤することができる。

【0018】注射剤としての製剤形態は、通常滅菌水水溶液を包含する。上記形態の製剤はまた緩衝剤・pH調節剤（リン酸水素ナトリウム、クエン酸等）、等張化剤（塩化ナトリウム、グルコース等）、保存剤（パラオキシ安息香酸メチル、p-ヒドロキシ安息香酸プロピル等）等の水以外の他の製薬補助剤を含有することができる。該製剤は細菌保持フィルターを通す沪過、組成物への殺菌剤の混入、組成物の照射や加熱によって滅菌することができる。該製薬はまた殺菌固体組成物として製造し、用時滅菌水等に溶解して使用することもできる。

【0019】経口投与剤は胃腸器官による吸収に適した形に製剤する。錠剤、カプセル剤、顆粒剤、細粒剤、粉末剤は常用の製剤補助剤、例えば結合剤（シロップ、アラビアゴム、ゼラチン、ソルビット、トラガカント、ポリビニルピロリドン、ヒドロキシプロピルセルロース等）、賦形剤（ラクトース、シュガー、コーンスターチ、リン酸カルシウム、ソルビット、グリシン等）、滑沢剤（ステアリン酸マグネシウム、タルク、ポリエチレングリコール、シリカ等）、崩壊剤（ポテトスターチ、カルボキシメチルセルロース等）、湿潤剤（ラウリル硫酸ナトリウム等）を包含することができる。錠剤は常法によりコーティングすることができる。経口液剤は水溶液等にしたり、ドライプロダクトにすることができる。そのような経口液剤は常用の添加剤例えば保存剤（p-ヒドロキシ安息香酸メチルもしくはプロピル、ソルビン酸等）を包含していても良い。

【0020】本発明のACE阻害剤中の本ペプチドの量は種々変えることができるが、通常1～100%（w/

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w）が適当である。本ACE阻害剤の投与量は有効成分として0．5～500mg／kg／dayが適当である。なお、本発明のペプチドの急性毒性はいずれもLD（ラット、経口投与）＞5g／kgである。

【0021】また、本発明のペプチドは多量に摂取しても生体に悪影響を与えない利点を有することから、そのまま、または種々の栄養分等を加えて、もしくは飲食品中に含有せしめて血圧降下作用、高血圧予防の機能を持たせた機能性食品、健康食品として食しても良い。すなわち、例えば各種ビタミン類、ミネラル類等の栄養分を加えて、例えば栄養ドリンク、豆乳、スープ等の液状の食品や各種形状の固形食品、さらには粉末状としてそのままあるいは各種食品へ添加して用いることもできる。かかる機能性食品、健康食品としての本発明のACE阻害剤中の本ペプチドの含有量、及び摂取量は上記製薬におけると同様で良い。

【0022】

【実施例】次に本発明を実施例により説明する。実施例中、%は重量%を示す。

実施例1　各オリゴペプチドの調製とACE阻害活性

a）　Val-His-Leu-Pro-Pro-Proの調製

ァーゼイン0．5gを蒸留水25mlに分散させ、1N　NaOHでpH12に調整しァーゼインを溶解させた。ついで限外沪過膜としてアミコン社PM-10（分画分子量10，000）を用いる限外沪過に付し、可溶化した低分子夾雑物を除去した。内液にpH12のNaOHを加え全容25mlとし、100℃で30分加熱しァーゼインを変性させた。この処理で低分子化した画分を除去するため再度上記と同じ限外沪過に付し、内液に蒸留水を加え全容25mlとし、さらに1N　HClで中性にした。

【0023】全容に対し0．25容の0．05M　CaCl含有0．25MトリスHCl緩衝液（pH8．5）を加え、37℃に保った後、サーモライシン（シグマ社）18mgを加えた。40時間後、1N　HClでpH1．7に調整して反応を停止させ、前記と同じ限外沪過に付して通過する低分子を回収した。これを1N　NaOHで中和後、濃縮した濃縮液をセファデックスLH-20のカラムに添加し蒸留水で溶出させた（溶出条件：カラム高さ70cm、内径1．6cm、試料添加量2ml、流速33ml／hr）。

【0024】各画分の少量を用いてHPLCによる溶出パターンを調べ、合成Val-His-Leu-Pro-Pro-Proが示す溶出位置と同位置のピークを持つ画分を回収した（HPLCの溶出条件：カラム　ウォーターズ社Radial　PAK　C-8、10μm、試料添加量5μl、流速1ml／min、溶出リン酸緩衝液（10mM　KHPO，50mM　NaSO，pH3．0）：アセトニトリル＝2：3、検出UV210nm）。

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【0025】この画分を5mM緩衝液（pH4．0）で平衡化したSP-トヨパール650Sカラムに添加し、0～0．3M　NaClの直線濃度勾配で溶出し、HPLCにて合成Val-His-Leu-Pro-Pro-Proと同位置に溶出されるピークを持つ画分を回収した（SP-トヨパール溶出条件：カラム高さ20cm、内径1．6cm、流速100ml／hr、溶出5mM酢酸緩衝液（pH4．0）を含む0～0．3M　NaCl，HPLCの溶出条件は前記と同じ）。

【0026】回収した画分を濃縮後、HPLCに付して合成Val-His-Leu-Pro-Pro-Proと同位置のピークのみを分取し、pH2のHClで洗浄した逆相シリカゲルカラムSepPAK　C-18（ウォーターズ社）に吸着させ、pH2のHClで混在する塩を除去した後、メタノールで溶出させ、アミノ酸分析を行った（分取時のHPLC溶出条件：試料添加量のみ25μlで他は最初の場合の条件と同じ）。上記でアミノ酸分析は試料を6N　HClに溶解し、真空下110℃で24時間加熱後アミノ酸分析計により行った。

【0027】この結果Leuを1としたモル比がVal　1．3、His　1．2、Leu1、Pro　3．1となり、Val-His-Leu-Pro-Pro-Proが回収できた。また、質量分析の結果は659（M+1）であり、上記ペプチドの予想分子量と一致した。

【0028】b）Leu-Pro-Pro-Proの調製

ロイシンアミノペプチダーゼ（ベーリンガーマンハイム山之内社）（5mg／ml液状）を0．05M　MgCl含有0．1Mトリス塩酸（pH8．6）800μlに溶解し酵素液とした。300μM　Val-His-Leu-Pro-Pro-Pro　50μlと酵素液200μlを混合し、37℃で23時間反応させた。反応後、反応液よりHPLCで合成Leu-Pro-Pro-Proと同位置に溶出されるピークを回収しアミノ酸分析を行った（HPLC溶出条件：使用するリン酸緩衝液のpHを2．5としたこと、及び試料添加量を10μlとした以外は最初の場合の条件と同じ）。この結果Leuを1としたモル比がVal　0．16、Leu　1、His0．19、Pro　2．51となり、Leu-Pro-Pro-Proが回収できた。

【0029】c）Leu-Pro-Proの調製

6．3mM　Leu-Pro-Pro-Pro　200μlと12N　HCl200μlを混合し、100℃で10分加水分解反応に服せしめた。ついでHPLCによる溶出で合成Leu-Pro-Proと同位置のピーク（3．18mm）を持つ画分が回収できた。（溶出条件：カラム　メルク社　Lichrosorb　RP-SelectB　5μm、流速1ml／min、溶出リン酸緩衝液（pH2．5）：アセトニトリル＝5：

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1、検出UV210nm)。

【0030】d) Val-His-Leu-Pro-Proの調製

Val-His-Leu-Pro-Pro-Proより上記c)と同様にして得た。

【0031】e) ACE阻害活性の測定

以上のようにして得た各ペプチドのACE阻害活性を以下のごとく測定した。すなわちまず、5gのラビットラングアセトンパウダーを50mlの0.1Mホウ酸ナトリウム緩衝液(pH8.3)に溶かし、40,000G、40分の条件下で遠心処理し、その上澄液をさらに上記緩衝液で5倍に希釈して、アンジオテンシン変換酵素液を得た。

【0032】各ペプチド溶液を試験管に0.03ml入れ、これに基質として、0.25mlのヒプリルヒスチジルロイシン(最終濃度5mM、NaCl 300mM含む)を添加し、ついで上記アンジオテンシン変換酵素液0.1mlを加え、37℃で30分間反応させた。そ*

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*の後、1N塩酸0.25mlを添加して反応を停止させた後、1.5mlの酢酸エチルを加え、酢酸エチル中に抽出されたヒプリル酸の228nmでの吸収値を測定し、これを酵素活性とした。なお、この条件で本発明阻害剤を含まない場合の228nmの吸収値はほぼ0.35であった。このような実験を複数行い、阻害率を次の式より算出した。

【0033】

【式1】

$$\text{阻害率} = \frac{A-B}{A} \times 100 \ (\%)$$

【0034】A:阻害剤を含まない場合の228nm吸収値

B:阻害剤添加の場合の228nm吸収値

そして、阻害率50%のときの阻害剤濃度Iを求めた。結果を表1に示す。

【0035】

【表1】

	I_{50}(μM)	備考
Leu-Pro-Pro	9.8	本発明
Val-His-Leu-Pro-Pro	18	比較例
Val-His-Leu-Pro-Pro-Pro	200	〃
Leu-Pro-Pro-Pro	(1mMで33%阻害)	〃

【0036】実施例2　Leu-Pro-Proの血圧降下作用

体重200gのWistar系雄性ラット(日本ラット(株)、1群5匹)をウレタン1.5g/kg腹腔内投与により麻酔し、常法に従って総頸動脈圧をトランスデューサー(SCK-590、日本光電(株))を介して連続的に記録した。下腿静脈より、生理食塩水に溶解したLeu-Pro-Proを投与し、その5、15、25および35分後にアンジオテンシンI(ヒト配列、シ※

※グマ社)100ng/kgを繰り返し投与して、前後の平均血圧の変化を測定した。対照としては、生理食塩水を投与したものを用いた。結果を表2に示す。本ペプチドは投与5分後のアンジオテンシンIによる昇圧を効果的に抑制し、その作用は35分後にもなお持続していた。

【0037】

【表2】

試験群	血圧上昇(△mmHg)			
	5分後	15分後	25分後	35分後
対照(生理食塩水投与群)	21±3 22±1	27±6 22±8	29±11 23±6	32±8 22±6
Leu-Pro-Pro 40mg/kg投与群	18±8 15±5	24±5 23±5	25±4 21±7	23±3 22±4
Leu-Pro-Pro 125mg/kg投与群	9±1 9±3	15±6 14±5	16±5 16±4	17±4 16±3

上段:最高血圧　下段:平均血圧

【0038】実施例3　静脈注射剤

Leu-Pro-Proを20～100倍(容積/重量)の滅菌生理食塩水に溶解し、無菌的にフィルター(孔径0.45μm)で沪過した沪液を注射剤とする。

【0039】実施例4　錠剤

Leu-Pro-Pro	7部
ヒドロキシプロピルセルロース	1部
ラクトース	10.9部
★ポテトスターチ	1部
ステアリン酸マグネシウム	0.1部

ヒドロキシプロピルセルロース1部を含む60%エタノール水溶液20部を調製し、本ペプチド7部およびラクトース10.9部を加えて充分に混練した後、減圧下で乾燥し、得られた乾燥物にポテトスターチ1部およびステアリン酸マグネシウム0.1部を加えて混和し、打錠機により製錠する。

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【0040】参考例1　γ-ゼインの調製　Esenの方法（J. Cereal Sci. 5, 117（1987））に準じて行った。粉砕とうもろこし（普通種デントコーン）100gに1%　2-メルカプトエタノールを含む60%イソプロピルアルコール水5倍量を加え、60℃で2時間撹拌することにより全ゼイン画分を抽出した。混合物を3，000Gで10分遠心分離し、上清に等容の蒸留水及び0．02容の3M酢酸ナトリウム水溶液を加え、少量の酢酸でpH6に合わせ4℃で一晩静置してαおよびβ-ゼインを沈殿させた。

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ついで3，000Gで10分遠心分離し、上清を凍結乾燥し、乾燥物を少量の蒸留水に分散させ、透析チューブを用いて蒸留水に対して透析し、ついで凍結乾燥して、淡黄色粉末としてγ-ゼイン0．4gを得た。

【0041】

【発明の効果】本発明によれば優れたACE阻害作用ならびに血圧降下作用を有するACE阻害剤が提供される。本発明のACE阻害剤は食品タンパク質由来のため大量に摂取しても極めて安全性が高く、従って副作用を示すこともない。

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